

AD_____

Award Number: DAMD17-99-1-9362

TITLE: Rational Design of Regulators of Programmed Cell Death in Human Breast Cancer

PRINCIPAL INVESTIGATOR: David Cowburn, Ph.D.

CONTRACTING ORGANIZATION: Rockefeller University
New York, New York 10021-6399

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010925 165

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 99 - 30 Jun 00)
4. TITLE AND SUBTITLE Rational Design of Regulators of Programmed Cell Death in Human Breast Cancer			5. FUNDING NUMBERS DAMD17-99-1-9362
6. AUTHOR(S) David Cowburn, Ph.D.			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Rockefeller University New York, New York 10021-6399 E-MAIL: cowburn@mail.rockefeller.edu			8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES Report contains color photos			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) The purpose of this research is to develop a better understanding of the intricate pathways of cell death and their contributions to breast cancers, with the goal of designing potential therapeutic agents for inducing or regulating apoptotic processes in breast cancer. The scope of this research has included the determination by NMR of the structure of the first pro-apoptotic BCL-2 like molecule (BID) and its analysis, identifying key structural issues associated with (a) the caspase catalyzed cleavage of part of BID (to produce a truncated form, 'tBID'), and (b) a likely structural model for the necessity of exposure of portion of one helix of BID and other homologues, in the BH3 motif. Current work focuses on efficient expression of the large quantities of BCL-2 family members for structural and biochemical assay, design and synthesis of BH3 mimics, and preliminary characterization of tBID in membrane bound form, prior to attempts to identify complexes of tBID for structural analysis. These studies have elucidated the structural basis for a set of molecular interactions that regulate programmed cell death, and allow the design of novel interventional agents that have investigative and therapeutic potential.			
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 21
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

DL Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

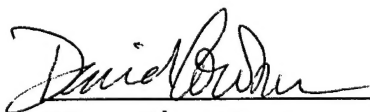
In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI - Signature

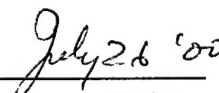

Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	5
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	8
References.....	8
Appendices.....	10,11,reprint

Introduction: This report covers activities related to the proposal from its submission, July 1, 1998 to date. The proposal was funded from July 1, 1999. The subject of the grant is the design of interventional agents for the biological process of apoptosis based on rational analysis of structures in the family of apoptotic and anti-apoptotic regulators. The purpose of the research is the development of a better understanding of the intricate pathways of cell death and their contributions to breast cancers, and represents a first step in designing potential therapeutic agents for inducing or regulating apoptotic processes in breast cancer. The scope of this research has included the determination by NMR of the structure of the first pro-apoptotic BCL-2 like molecule (BID) and its analysis, identifying key structural issues associated with (a) the caspase catalyzed cleavage of part of BID (to produce a truncated form, 'tBID'), and (b) a likely structural model for the necessity of exposure of portion of one helix of BID and other homologues, in the BH3 motif. Current work focuses on efficient expression of the large quantities of BCL-2 family members for structural and biochemical assay, design and synthesis of BH3 mimics, and preliminary characterization of tBID in membrane bound form, prior to attempts to identify complexes of tBID for structural studies.

Body: *The solution structure of BID.* Programmed cell death is a highly evolutionarily conserved biological process critical for development and homeostasis in all multicellular organisms (1, 2). This process allows for the removal of redundant or damaged cells, playing a vital role in normal cellular development, tissue homeostasis, and immunological defense (3, 4). Dysregulated programmed cell death can contribute to cancer, autoimmune disease and neurodegenerative disorders (5, 6). Developmental or environmental signals can regulate signals for either cell death or cell survival. The study of complex signaling pathways of programmed cell death has led to the identification of a large number of molecules involved in regulating apoptotic death, or promoting cell survival (7, 8).

Key features of programmed cell death include a cascade of proteases tightly controlled by apoptotic signaling pathways. As part of a critical apoptotic pathway regulating a critical checkpoint in mitochondria, the Bcl-2 family of proteins comprises both anti- and pro-apoptotic regulators (5). The founding member of this family, Bcl-2, was first identified on the basis of its involvement in B-cell malignancies, where chromosomal translocations resulted in overexpression of Bcl-2 and an increased resistance to programmed cell death (9, 10). This discovery established a new class of oncogenes, for which extended cell survival and resistance to apoptosis results in increased oncogenic potential (11).

Membership in the Bcl-2 family is defined based on homology to at least one of four conserved sequence motifs known as Bcl-2 homology motifs, BH1 to BH4 (sometimes referred to as domains). Most anti-apoptosis family members have BH1 and BH2 motifs and many have all four BH motifs. In contrast, many of the pro-apoptotic family members (Bid, Bad, Bik, Bim, Blk, Hrk) only possess the conserved BH3 motif. The ratio of anti- and pro-apoptotic molecules apparently determines whether a cell will respond to a proximal apoptotic stimulus. This competition is mediated, at least in part, by competitive dimerization between anti and pro-apoptotic pairs (5).

An important sub-family within the Bcl-2 family consists of pro-apoptotic molecules with homology only in the BH3 region (12). The BH3-only class of cell death effectors are conserved components of a central death pathway. Studies of developmentally

regulated cell death have revealed the BH3-only molecule Egl-1 plays a major role in controlling programmed cell death in *C. elegans* by acting as a negative regulator of the Bcl-2-like cell death inhibitor CED-9 (13). The Bcl-2 family member Bid belongs to the BH3-only class of pro-apoptotic killers. Like other members of this class of molecules, Bid shows no recognizable sequence homology to BH1, BH2 or BH4 domains, and requires a functional BH3 domain for its dimerization and pro-apoptotic activity (14, 15, 16). Very recently it has been reported that caspase-8 mediates the cleavage of an inactive 23kD cytosolic Bid to produce a truncated 15kD fragment (referred to as tBid) that translocates to the mitochondria causing cytochrome c release, activation of caspases and the final commitment to cell death (17, 18, 19). In this work, we determined the solution structure of Bid using NMR spectroscopy. This work provides the first structure of a pro-apoptotic Bcl-2 family member (simultaneously with a paper from Wagner's Laboratory (20)) and offers insight into the structural basis of the pro-apoptotic function of the Bcl-2 family death agonists. Based on sequence/structure similarity or dissimilarity to Bid the mechanisms of other pro-apoptotic family members were proposed, illustrating a fold class including both Bid and the previously determined Bcl-X_L structure (21, 22). From the likely structure of tBid, a structural mechanism for Bid and other pro-apoptotic family members was suggested. The paper resulting from this work (23) is attached.

Model of Bid mediated apoptosis

The exposure of the BH3 domain in Bid, and the pro-apoptotic function of the molecule, is regulated through a post-translational cleavage event. Other mechanisms of post-translational modification regulate activities of other family members (16, 24). This type of regulation allows the rapid response necessary for apoptosis.

The structure of Bid sheds light on its role as a death agonist, and the requirements for pro-apoptotic action in the Bcl-2 family. What makes a Bcl-2 family member a death agonist? It appears that two criteria must be met for pro-apoptotic function. The first requirement is one of cellular localization. There is strong evidence for several Bcl-2 family members that mitochondrial translocation is a key determinant for death inducing activity. Bid (17, 18, 19) and Bax (25) are inactive as cytosolic components but induce death upon their targeting to mitochondria. The activity of Bad, on the other hand, is down-regulated by phosphorylation which sequesters the protein in the cytosol away from mitochondria targets (16). The second, and perhaps more critical, criteria for death agonism is the exposure of the BH3 surface. The cleavage of Bid by caspase-8 is likely to expose a BH3 motif that is buried in the uncleaved molecule. Based on our structure predictions for the Bcl-2 family some members of the BH3-only class appear to have their BH3 motif exposed, or at least not under the same conformational constraints as other members of the Bcl-2 family. Molecules possessing an exposed BH3 motif (Egl-1, Hrk, Bik, Bim, tBid) might be expected to be constitutively active death agonists. While other members of the family possessing a 'hidden' BH3 motif are regulated by post-translation modification events (i.e., cleavage, phosphorylation) that expose the BH3 epitope (24, 26, 27) that induce their pro-apoptotic function. The structural work then strongly supported the original hypothesis that BH3 recognition was a key event in pro-apoptotic action, and suggested that the exposure of the hydrophobic face may be key.

The expression of BCL-2 family members. The work described above involved a collaboration with Professor S. Korsmeyer, who had provided the expressed protein.

He subsequently gave us *E. coli* with the plasmid expressing the GST-BID fusion protein, and the expression of the protein was tested and BID prepared in this laboratory. Since the research program seeks to investigate the biochemical interactions of BID, tBID, other BCL-2 family members and possible binding partners, we undertook to express optimally several of these which are tabulated in Table 1 (Appendix). These have originally obtained from the Korsmeyer or Marie Hardwick labs.

BH3 mimics. As outlined in the original proposal, we wished to design and test a conformationally constrained α helix mimicking the full BH3 motif of BID and/or other proapoptotic members of the BCL-2 family. We implemented the strategy of using 1,1+7 side chain links for the sequence NIAKHLAQIGDEMD, with the K...D linkage being obtained by intermediate protection with allyl-K and Alloc-E (see Fig 2 Appendix). Side chain deprotection was obtained by hydrogenation with Pd, and cyclization by use of the procedure of Phelan (28). The product was characterized by EI-MS to the nominally correct mass, and its CD spectrum is shown in Fig. 3. While α helicity is apparent in the spectrum, the magnitude of the 223 nm minimum is less than the expected c. -30,000 (~68%), and may reflect time averaging of the structure between an α helix and more random structures. Surface plasmon resonance and other affinity measurements to BCL-2 family members are in progress.

Structure of membrane bound tBID. After caspase 8 cleavage of BID to tBID, the truncated form is very water insoluble, and is generally thought to be recruited to the mitochondrial membrane in the cell. As is widely recognized, structural characterization in the membrane or membrane-like environment presents considerable difficulties to current methods, either crystallographically or by NMR. We chose to attempt to characterize the tBID structure in membrane mimics or aqueous detergents or in organic solvents. Using sodium dodecyl sulfate micelles (previously used by us in identifying the recognition of PH domains by phospholipids (29)), CD spectra suggest that tBid is ~ 80% helical consistent with no overall change in secondary structure after the BID->tBID cleavage. Since this was unlikely to be controversial, we deferred further structure determination attempts until tBID complexes with mimics, or other BCL-2 family members could be prepared, and this is now in progress. We expect that this will be significantly enhanced by use of the HPA BiaCore SPR chips and selective assay with phospholipids common to the mitochondrial membrane.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Solution structure of BID
- 2) Development of hypothesis for the sequence basis of anti- & pro-apoptotic BCL-2 family members, and the role of post translation modification in their conversions.
- 3) Design and synthesis of stabilized BH3 mimic.

REPORTABLE OUTCOMES:

Manuscripts, abstracts, and presentations:

- 1) J. M. McDonnell, D. Fushman, C. L. Milliman, S. J. Korsmeyer, D. Cowburn, *Cell* **96**, 625-34 (1999). Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

2) Presentation, Jan 1, 1999, James McDonnell, University of Cambridge, UK, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

3) Presentation, Mar, 1999. JM, Cell Death Club, NY, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

4) Presentation, July 14, 1999, David Cowburn, New York Structural Biology Discussion Group, Cold Spring Harbor. Including - Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

5) Presentation, October, 1999. JM. Johns Hopkins School of Medicine, Dept. Pharmacology, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

6) Presentation, October, 1999. JM. Columbia University Coll. P&S., Dept. Pharmacology, Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists.

7) Presentation, Jan 2000, DC. City College of New York Structural Biology Symp. Degrees granted. None.

Development of cell lines, tissue or serum depositories. None.

Informatics such as database and animal models, etc. None.

Funding applied for based on work supported by this award. None.

Employment or research opportunities applied for and/or received on experience/training supported by this award. Dr. James McDonnell, supported by the award, will become Lecturer in Molecular Biophysics, University of Oxford, UK, and Fellow of Somerville College, in September.

Conclusions. We are now well positioned by structural and synthetic progress to develop biochemical binding assays for the BCL-2 family, to design rationally synthetic ligands for this interaction, and to investigate further the structure of pro-apoptotic complexes.

References.

1. M. C. Raff, *Nature* 356, 397-400 (1992).
2. R. E. Ellis, J. Y. Yuan, H. R. Horvitz, *Annu Rev Cell Biol* 7, 663-98 (1991).
3. A. Strasser, D. C. Huang, D. L. Vaux, *Biochim Biophys Acta* 1333, F151-78 (1997).
4. C. B. Thompson, *Science* 267, 1456-62 (1995).
5. D. T. Chao, S. J. Korsmeyer, *Annu Rev Immunol* 16, 395-419 (1998).
6. J. C. Reed, *Adv Pharmacol* 41, 501-32 (1997).
7. S. Cory, *Annu Rev Immunol* 13, 513-43 (1995).
8. E. Yang, S. J. Korsmeyer, *Blood* 88, 386-401 (1996).
9. A. Bakhshi, et al., *Cell* 41, 899-906 (1985).
10. Y. Tsujimoto, J. Cossman, E. Jaffe, C. M. Croce, *Science* 228, 1440-3 (1985).
11. D. L. Vaux, S. Cory, J. M. Adams, *Nature* 335, 440-2 (1988).
12. A. Kelekar, C. B. Thompson, *Trends Cell Biol* 8, 324-30 (1998).
13. B. Conradt, H. R. Horvitz, *Cell* 93, 519-29 (1998).
14. K. Wang, X. M. Yin, D. T. Chao, C. L. Millman, S. J. Korsmeyer, *Genes Dev* 10, 2859-69 (1996).
15. A. Kelekar, B. S. Chang, J. E. Harlan, S. W. Fesik, C. B. Thompson, *Mol Cell Biol* 17, 7040-6 (1997).

16. J. Zha, H. Harada, E. Yang, J. Jockel, S. J. Korsmeyer, *Cell* 87, 619-28 (1996).
17. H. Li, H. Zhu, C. J. Xu, J. Yuan, *Cell* 94, 491-501 (1998).
18. X. Luo, I. Budihardjo, H. Zou, C. Slaughter, X. Wang, *Cell* 94, 481-90 (1998).
19. A. Gross, et al., *J Biol Chem* 274, 1156-63 (1999).
20. J. J. Chou, H. Li, G. S. Salvesen, J. Yuan, G. Wagner, *Cell* 96, 615-24 (1999).
21. S. W. Muchmore, et al., *Nature* 381, 335-41 (1996).
22. M. Sattler, et al., *Science* 275, 983-6 (1997).
23. J. M. McDonnell, D. Fushman, C. L. Milliman, S. J. Korsmeyer, D. Cowburn, *Cell* 96, 625-34 (1999).
24. E. H. Cheng, et al., *Science* 278, 1966-8 (1997).
25. I. S. Goping, et al., *J Cell Biol* 143, 207-15 (1998).
26. L. C. Lee, J. J. Hunter, A. Mujeeb, C. Turck, T. G. Parslow, *J Biol Chem* 271, 23284-8 (1996).
27. J. J. Hunter, T. G. Parslow, *J Biol Chem* 271, 8521-4 (1996).
28. J. C. Phelan, N. J. Skelton, A. C. Braisted, R. S. McDowell, *J. Am. Chem. Soc.* 119, 455-460 (1997).
29. J. Zheng, et al., *J. Mol. Biol.* 255, 14-21 (1996).

Table 1. BCL-2 family constructs expressed for future studies.

<u>Protein</u>	<u>Purification tag</u>	<u>Vector</u>	<u>Comments</u>
BID	GST	pGEX-3X	construct used for protein labeling and structure determination
BID	His-tag	pET-5B	
tBID	GST	pGEX-3X	construct used for micellar and membrane bound BID studies
tBID	His-tag	pET-5B	
BCL-X _L	GST	pGEX-3X	
tBCL-X _L	His-tag	pET-5B	
BAX	---	pSG5	
BCL-2	---	pSG5	

Figure 1. Model of BH3 mimic

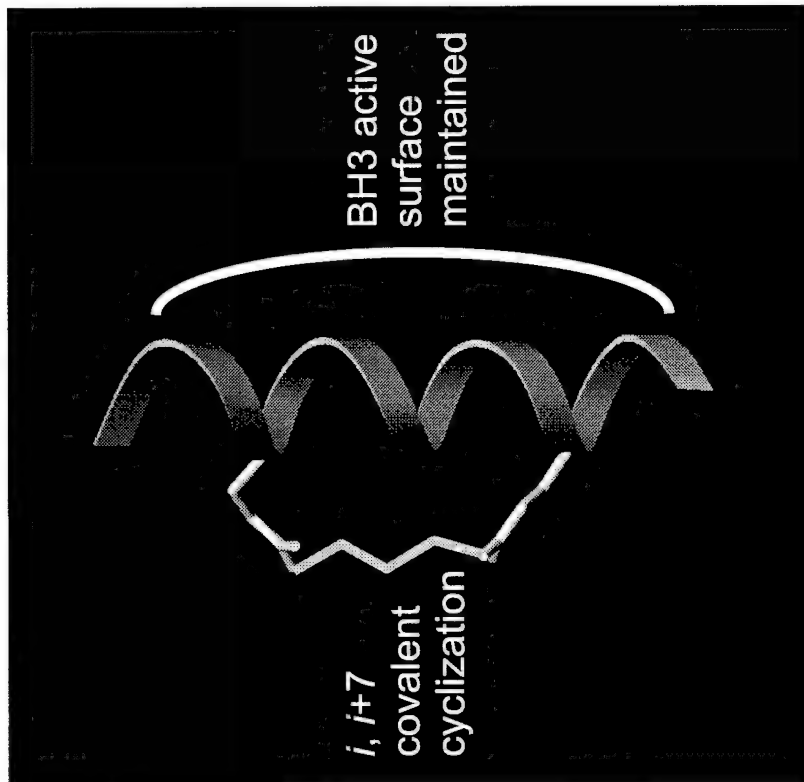
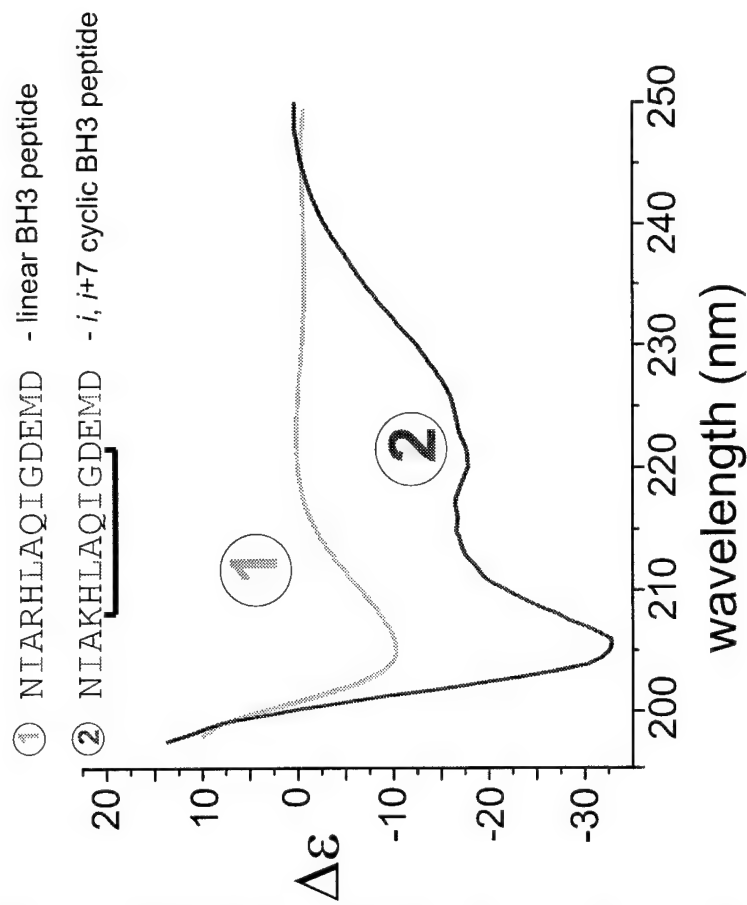


Figure 2. CD of synthetic BH3 peptides



Solution Structure of the Proapoptotic Molecule BID: A Structural Basis for Apoptotic Agonists and Antagonists

James M. McDonnell,* David Fushman,*
Curt L. Milliman,† Stanley J. Korsmeyer,†§
and David Cowburn*‡

*The Rockefeller University
New York, New York 10021

†Howard Hughes Medical Institute
Washington University School of Medicine
St. Louis, Missouri 63110

Summary

Members of the BCL2 family of proteins are key regulators of programmed cell death, acting either as apoptotic agonists or antagonists. Here we describe the solution structure of BID, presenting the structure of a proapoptotic BCL2 family member. An analysis of sequence/structure of BCL2 family members allows us to define a structural superfamily, which has implications for general mechanisms for regulating proapoptotic activity. It appears two criteria must be met for proapoptotic function within the BCL2 family: targeting of molecules to intracellular membranes, and exposure of the BH3 death domain. BID's activity is regulated by a Caspase 8-mediated cleavage event, exposing the BH3 domain and significantly changing the surface charge and hydrophobicity, resulting in a change of cellular localization.

Introduction

Programmed cell death is a highly conserved biological process critical for all multicellular organisms (Ellis et al., 1991; Raff, 1992). This process allows for the removal of noninstructed, misinstructed, as well as damaged cells, playing a critical role in embryonic development, the maintenance of tissue homeostasis, and immunological defense (Thompson, 1995; Strasser et al., 1997). Dysregulated programmed cell death can contribute to cancer, autoimmunity, immunodeficiency, infertility, and neurodegenerative disorders (Chao and Korsmeyer, 1998). Developmental or environmental cues deliver complex signals that promote cell death or survival. Dissection of the cell death pathway has led to the identification of a large number of both pro- and antiapoptotic molecules (Adams and Cory, 1998).

The BCL2 family of proteins constitutes a critical decisional checkpoint within the common portion of the apoptotic pathway, upstream to the irreversible damage to cellular constituents. The BCL2 family is comprised of both proapoptotic and antiapoptotic molecules (Adams and Cory, 1998; Chao and Korsmeyer, 1998). Full members of the BCL2 family share homology in three or four conserved domains entitled BH1-4. The ratio of pro-

(BAX, BAK, and BOK) versus antiapoptotic (BCL2, BCL-X_L, BCL-W, MCL1, and A1) members determines in part how cells respond to proximal death and survival signals (Oltvai et al., 1993). Mutagenesis of BCL2 indicated that intact BH1 and BH2 domains were required to interact with BAX and to repress cell death (Yin et al., 1994). Moreover, deletions within BAK (Chittenden et al., 1995) and an extensive mutagenesis of BAX (Wang et al., 1998) indicated that the amphipathic BH3 domain of proapoptotic molecules was critical for dimerization and death.

A divergent subset of the BCL2 family possesses substantial sequence homology only within the conserved BH3 domain. These "BH3 domain only" members include the mammalian BID, BAD, BIK, BIM, BLK, and HRK, all of which are proapoptotic. This lends credence to the thesis that BH3 represents a minimal death domain (Boyd et al., 1995; Yang et al., 1995; Wang et al., 1996; Inohara et al., 1997; Hegde et al., 1998; O'Connor et al., 1998). The identification of a "BH3 domain only" member in *C. elegans*, EGL-1, argues that this class of death effectors is a conserved component of a central death pathway (Conradt and Horvitz, 1998). Like other members of this class of molecules, BID shows no recognizable sequence homology to BH1, 2, or 4 domains and requires the BH3 domain for its heterodimerization with BAX, BCL2, or BCL-X_L (Wang et al., 1996). Mutational analysis indicated that an intact BH3 domain, perhaps one that retained interaction with BAX, was also required for the apoptotic activity of BID.

Evolving evidence suggests that the proapoptotic BCL2 members may exist in inactive and active forms. BAX, despite possessing a hydrophobic C terminus, exists in the soluble fraction of cells as well as in mitochondrial membranes. Death stimuli ranging from staurosporine to the withdrawal of survival factors result in the translocation of monomeric BAX from the cytosol to mitochondria, where it is apparently a homodimeric integral membrane protein (Wolter et al., 1997; Gross et al., 1998). The "BH3 domain only" molecules are attractive candidates to connect proximal signal transduction events with the distal death effector mechanism by their posttranslational modification. For example, BAD is inactivated by phosphorylation in response to IL-3 survival factor signaling, having a dual impact on BAD's location and its binding partners. Phosphorylated BAD is sequestered in the cytosol bound to 14-3-3, whereas only the active, nonphosphorylated BAD is capable of heterodimerization with BCL-X_L at membrane sites, thereby preventing cell death (Zha et al., 1996). Recently, BID has been reported to be activated by proteolytic cleavage by an activated Caspase 8 in response to treatment with TNF α or anti-FAS. The "inactive" p22 cytosolic BID is cleaved at a caspase recognition site to produce a major p15 truncated fragment, referred to as tBID, that translocates to mitochondria (Li et al., 1998; Luo et al., 1998; Gross et al., 1999). The p15 tBID is capable of targeting mitochondria, where it becomes an integral membrane protein.

Thus it remains critical to determine the three-dimensional structure of a proapoptotic BCL2 family member

‡To whom correspondence should be addressed (e-mail: cowburn@rockvax.rockefeller.edu).

§Present address: Dana-Farber Cancer Institute, Harvard Medical School, 1 Jimmy Fund Way, Boston, Massachusetts 02115.

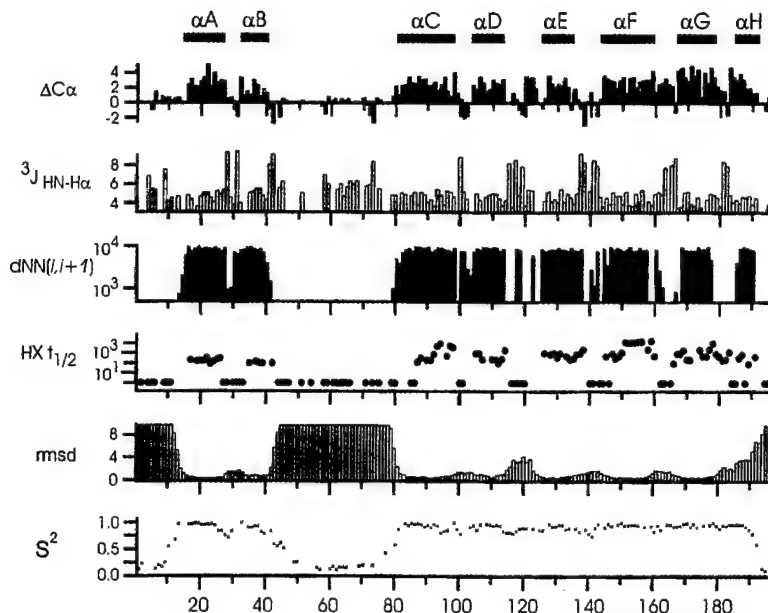


Figure 1. BID Assignment and Secondary Structure Summary

The secondary $^{13}\text{C}\alpha$ chemical shift ($\Delta C\alpha$) (Wishart et al., 1992), $^3J_{\text{HN-H}\alpha}$ (Wüthrich, 1986), and $d_{\text{NN}}(i, i+1)$ NOE correlations (normalized to a maximum intensity of 10^4) give strong signals characteristic of helical structure in the indicated regions. The hydrogen-deuterium exchange rates (HX) for backbone amides are strongly protected in the helical elements. The rmsd values are the pairwise values of 20 structures. S^2 is the squared order parameter for the ^{15}N - ^1H amide of each residue and is a direct measure of conformational flexibility on the nanosecond-picosecond timescale; $S^2 = 1$ in the absence of NH bond reorientations, while $S^2 = 0$ corresponds to a completely unrestricted motion of the bond.

to provide insight into how this subset of molecules mediates cell death rather than survival. Multiple models have been postulated to account for their prodeath activity. The characteristics of "BH3 domain only" molecules suggest they may serve as death ligands, sensors that receive death signals in the cytosol and translocate to membranes where they bind to either active or inactive BCL2 members, serving as receptors (Wang et al., 1996). An alternative theory of displacement holds that these proapoptotic molecules might displace Apaf1 from the BCL-X_L pocket to activate a caspase cascade (Chinnaiyan et al., 1997). p15 tBID's role as an integral membrane protein raises the possibility that it might also play a role as a pore former (Antonsson et al., 1997; Schlesinger et al., 1997). Moreover, while the "BH3 domain only" molecules have a short sequence homology in the BH3 motif, it is essential to delineate the other parts of the molecular structures. Here we report the solution structure of BID using NMR spectroscopy, providing the structure of a proapoptotic BCL2 family member and offering insight into the structural basis of the proapoptotic function of the BCL2 family death agonists. Based on sequence/structure similarity or dissimilarity to BID, the mechanisms of other proapoptotic family members are proposed, illustrating a fold class including both p22 BID and the previously determined BCL-X_L structure (Muchmore et al., 1996; Sattler et al., 1997). The structural changes caused by removing the N terminus to produce p15 tBID suggest a mechanism for proapoptotic activity of these family members.

Results

Structure Determination of BID

The structure of the full 22 kDa BID protein (residues 1–195) was determined using heteronuclear NMR spectroscopy. Initial ^1H - ^{15}N HSQC maps showed a high degree of spectral overlap at low temperatures. Since the BID protein was found to be exceptionally thermostable,

all of the NMR experiments for assignment and structure calculations were performed at 45°C with increased effective resolution. Backbone and side-chain assignment was carried out using both the CBCA (Grzesiek and Bax, 1993) and NOE (Wüthrich, 1986) based assignment strategies. Some residues at the unstructured N terminus (residues M1–S2) and in a large flexible loop (residues R31–E33, E52–Q56, and N68–G70) were not assigned. Also, the correlations for several residues found in loops were obtained primarily from the CBCA-based assignment approach, as line broadening from chemical exchange made assignment of individual amide NOEs difficult. Three sets of triple-resonance experiments (described in Experimental Procedures) were used to define the backbone assignments. Additionally, extensive sets of experiments were performed to analyze BID dynamics and hydrodynamics.

The structure of the BID protein was defined based on ~1700 NMR-derived distance constraints. This included 1350 structurally relevant NOE-based constraints, 275 dihedral angle constraints measured from J-coupling constants and local bond geometry (Güntert et al., 1991), and constraints from 56 hydrogen bonds implied by hydrogen-deuterium exchange experiments and local secondary structure. This corresponds to an average density of ~15 constraints per residue in all regions of secondary structure.

Structure Description of BID

The three-dimensional structure of BID consists of eight α helices arranged in three layers (Figure 2). Two central hydrophobic helices (αF and αG) are surrounded by amphipathic helices on either side. There is a large disordered loop between helices B and C; analysis of backbone dynamics (S^2 values in Figure 1) gives a direct measure of flexibility and confirms the conformational mobility of the region. The C-terminal helix (αH) has well-defined local structure, but no long-range NOEs are observed, so the helix is poorly defined relative to the

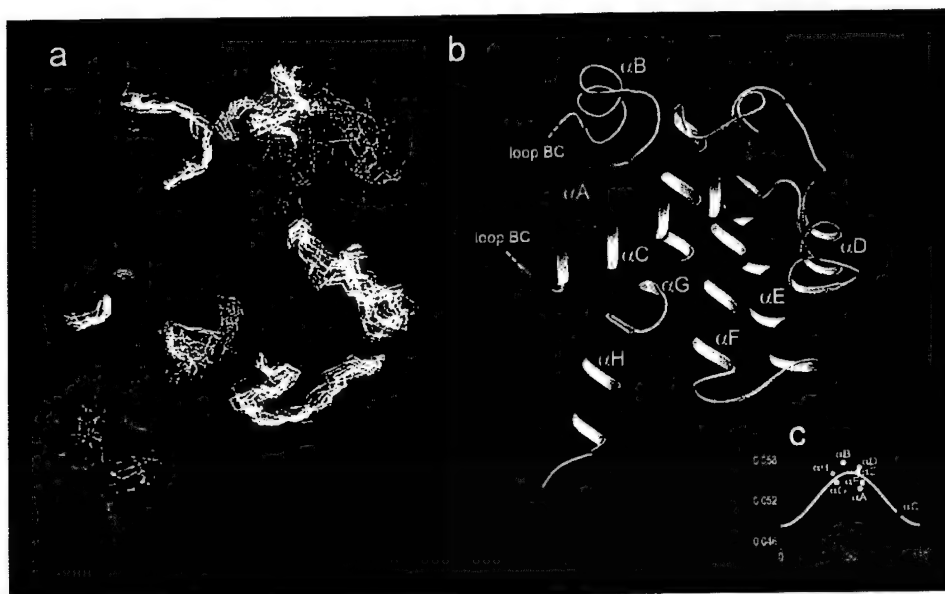


Figure 2. The Three-Dimensional Structure of BID

(a) View of the backbone (N, C α , C') of 20 superimposed NMR-derived structures of BID. Helical elements are colored in red and loops in white; the flexible amino terminus and the BC loop are not shown.

(b) A ribbon diagram of BID; helical segments are labeled. The figure was generated using SETOR (Evans, 1993).

(c) The average orientation of individual helices as calculated using anisotropic relaxation analyses. The x axis shows the angle θ : the orientation axis of each helix, calculated from the 20 NMR-derived structures, relative to the unique principal axis of the overall rotational diffusion tensor. On the y axis is the value $(2R_2'/R_1' - 1)^{-1}$, derived from relaxation measurements. The white line is the theoretical relationship between these two parameters for a D_x/D_y ratio of 1.18. The individual values for helices αA , αB , αD , αE , αF , and αG are shown in yellow, helix αH in green, and helix αC in red.

rest of the structure. However, its position in Figures 2a and 2b is fully supported by calculations of helix orientation by analysis of relaxation data. Because BID is hydrodynamically anisotropic, the longitudinal (R_1) and transverse (R_2) relaxation rates provide structural orientation information (Broadhurst et al., 1995; Tjandra et al., 1997) (described in Experimental Procedures). The results of this analysis (Figure 2c) suggest that helix αC is nearly parallel to the unique principal axis of the overall rotational diffusion tensor, while all the other helical segments are nearly perpendicular to this. This type of measurement acts as an independent confirmation of the NMR structures calculated using the traditional NOE-based strategies. The global rms deviations for the first seven helices (αA – αG) are 0.7 Å for backbone atoms (see Figure 1; further structure statistics are given in Experimental Procedures). A bundle of the 20 lowest target function NMR conformers and a ribbon diagram depicting the elements of secondary structure are shown in Figures 2a and 2b, respectively.

Structural Comparison

Visual inspection clearly places the BID structure in the SCOP classification fold class of toxins' membrane translocation domains (Murzin et al., 1995). Despite the very low sequence homology between the two molecules, the overall tertiary fold derived for BID is similar to that observed for the BCL-X_L structure (Muchmore et al., 1996; Sattler et al., 1997). This has independently been observed by Chou et al. (1999 [this issue of *Cell*]), who also solved the solution structure of BID. This level

of structural similarity was unexpected based on sequence comparisons. Compared with BCL-X_L, BID has an additional helix (αB) before the disordered loop. This helix is well defined and packs against αA and the FG loop. A comparison with the BCL-X_L structure reveals a single helical turn in the analogous position (residues W24–V30 in BCL-X_L). Several other members of the BCL2 family also have a predicted possible helix in this position, so perhaps a short αB helix is a general characteristic for this structural class of the BCL2 family. Rmsds between BID and BCL-X_L (PDB code 1maz) (4.90 Å over 69 residues and seven helices) and BID and diphtheria toxin (PDB code 1sgk) (4.13 Å over 38 residues and four helices) are consistent with distantly related proteins belonging to the same structural class. Exposure of surface components are, however, quite different (see Discussion).

Structure Predictions for the BCL2 Superfamily

Having solved the BID structure, we asked what general conclusions could be drawn for sequence/structure relationships within the BCL2 family. We performed primary sequence alignments for family members belonging to the different BCL2 subfamilies. Because primary sequence analysis had failed to predict any structural similarity between BID and BCL-X_L outside of the BH3 motif (Wang et al., 1996; Adams and Cory, 1998), we also performed predictions of secondary structure for family members. Patterns of secondary structure are often more sensitive for structure prediction than primary sequence comparison alone. For the sequence

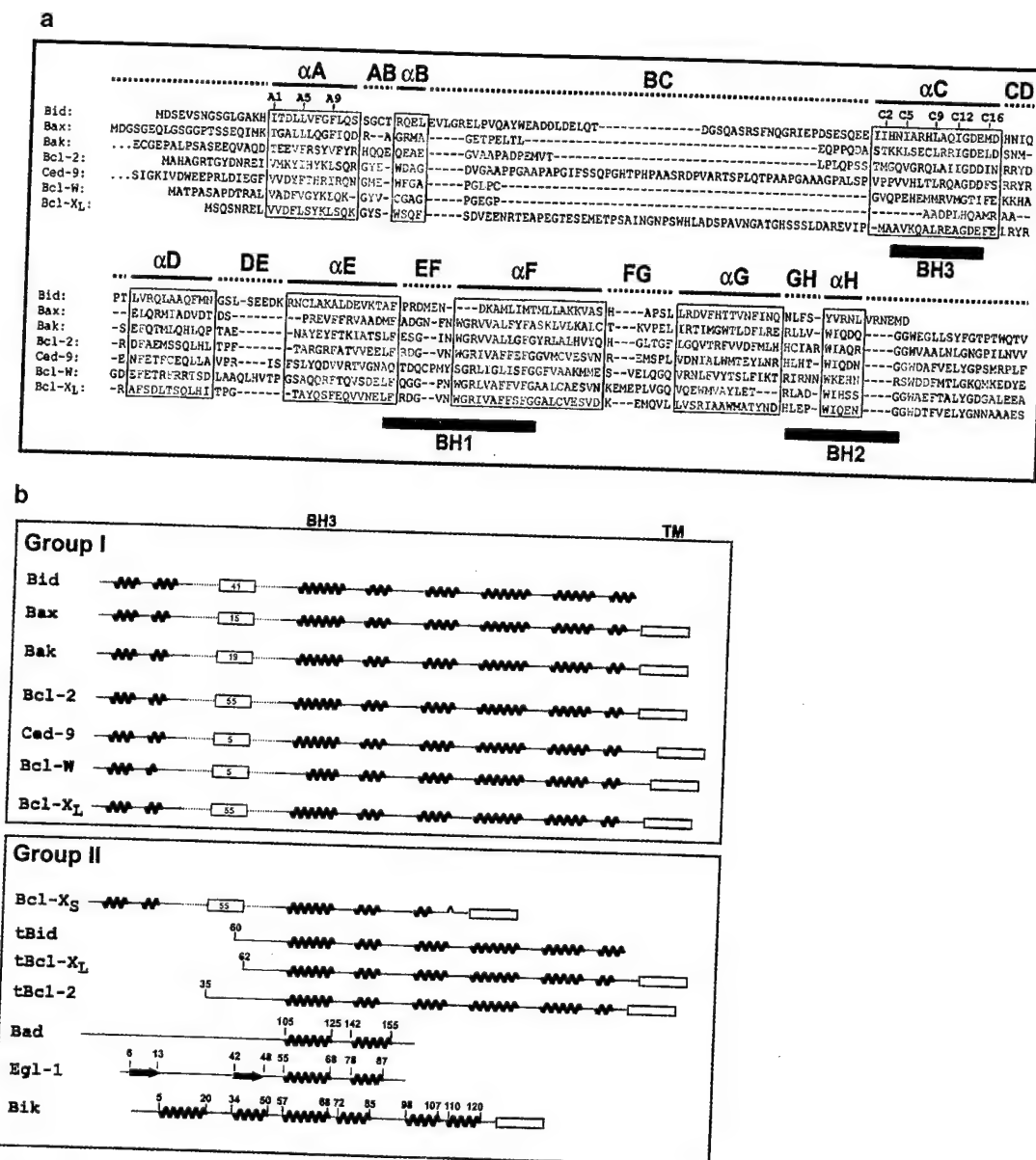


Figure 3. Sequence Alignment of BID with Other BCL2 Family Members and Secondary Structure Predictions for a Representative Set of BCL2 Family Members

(a) Boxed regions represent the consensus helical elements, and BH regions are indicated. The principal consensus elements are based on the BID structure; some helices, especially the short αB in BCL-X_L, only approximately concur with observed ϕ , ψ angles. Residues in blue are the helical elements in BID, while residues in red are the helical elements in BCL-X_L. The green residues illustrate the conserved hydrophobic repeat characteristic for amphipathic helices, implying a conservation of topology for this structural subclass of the BCL2 family. Note that the 3-4 hydrophobic repeat is not present in helix αF because this is the central core helix and is not amphipathic. Additional sequence alignments of other family members are available elsewhere (Chittenden et al., 1995; Muchmore et al., 1996).

(b) The proteins in this figure were chosen as a representative set of BCL2 family members. Helix αB is unusually long in BID compared with other family members. The length of the BC loop is indicated by the boxed value. The red carat symbol in BCL-X_S denotes the site of a 63-amino-acid deletion compared with BCL-X_L. Boundaries of secondary structure elements are often difficult to define. It is expected that there is variability in these boundaries for the different family members; the helical representations in the figure are not meant to precisely define boundaries in the group II family should also be considered approximate.

alignment of the BCL2 family, we used the program CLUSTALW (Thompson et al., 1994) and aligned by hand where CLUSTALW failed. Low levels of sequence similarity in this family made completely automated alignments impractical. The aligned sequences were used

for secondary structure predictions for the various family members, and where possible, the consensus from the multiple approaches was used.

Based on sequence and secondary structure alignments, members of the BCL2 family can be grouped into

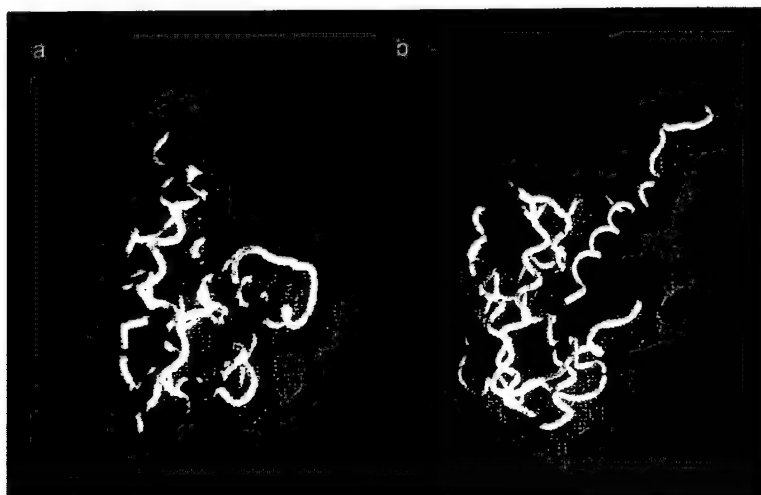


Figure 4. Comparison of BID and BCL-X_L BH123 Acceptor Region

(a) The BID and (b) BCL-X_L-BAK complex (PDB code 1xl) [Sattler et al., 1997] structures were colored according to surface hydrophobicity (red for hydrophobic, white for hydrophilic) using GRASP (Nicholls et al., 1991). The backbone worms for both BID and the BCL-X_L-BAK BH3 peptide complex are colored yellow.

two main structural categories. Group I family members have a sequence pattern consistent with the conservation of the BID/BCL-X_L topology. The members of group I, including the proapoptotic molecules BID, BAX, and BAK, will have their BH3 domain buried, and therefore must rely on some mechanism of conformational change to induce their proapoptotic activity. When one considers the timescale on which apoptosis occurs—apoptotic cell death generally takes less than 60 min [Thornberry and Lazebnik, 1998]—a posttranslational modification that serves as the “on switch” appears to be the most realistic mechanism for structural and/or conformational activation. The group II BCL2 family members are either truncated group I molecules (tBID, tBCL2, tBCL-X_L, BCL-X_S) or family members that have an unrelated tertiary structure (e.g., BAD); this class of family members seems likely to have their BH3 domains surface exposed and consequently, in contrast with BID, would be constitutively active.

Discussion

Implications of the BID Structure and Caspase 8 Cleavage

BID does not form homodimers, nor does it serve as an acceptor for the BH3 motifs from other BCL2 family members [Wang et al., 1996], because it lacks the groove formed by the BH motifs (called BH1, 2, 3) that is the “acceptor” pocket of the BH3 binding event. Not only does BID lack the strong primary sequence homology to BH1 and BH2 motifs found in the full members of the family (Figure 3a), but the tertiary structure of the region is quite different than that of BCL-X_L, sufficient to preclude the binding of a BH3 donor segment. Specifically, compared with the BCL-X_L structure, and by analogy to BCL2 and BAX molecules, BID has a well-formed helix at position α D that runs nearly antiparallel with α E; the open groove seen in BCL-X_L in this region is not present (see Figure 4). Furthermore, BID has a deletion in the EF loop (the BH1 region) (Figure 3a). While BCL-X_L exposes a deep cleft of marked hydrophobicity, BID has no such character in this region. The ability to form homodimers or heterodimers through the BH1, 2, 3 cleft

appears to be critical to the function of the antiapoptotic molecules [Yin et al., 1994; Chittenden et al., 1995]. If dimerization with BCL2 family members through this cleft is an important characteristic for death antagonism, it is clear why BID cannot function in an antiapoptotic manner.

It is the hydrophobic surface of the amphipathic BH3 helices that mediates BCL2 dimerization events [Sattler et al., 1997; Zha et al., 1997]. In the BID structure, this hydrophobic face of the BH3 helix (the α C residues indicated in Figure 3a) is mostly buried and less available for avid interaction with BCL2 or other binding partners. Presented as the whole 22 kDa protein, BID is predominantly inactive. Upon cleavage by Caspase 8, BID becomes an active p15 death-inducing product. In direct binding experiments between BID/tBID and BCL-X_L, it was demonstrated that in the absence of detergents tBID binds BCL-X_L avidly, while a BID-BCL-X_L complex could not be detected [Li et al., 1998]. Caspase 8 cleaves cytosolic BID predominantly at D59 to yield a 15 kDa proapoptotic fragment. D59 resides in the unstructured loop (D59 $S^2 = 0.15$) and would be predicted to be completely available to proteolytic cleavage. The 15 kDa proapoptotic fragment loses helices α A and α B and a portion of the unstructured loop. Loss of these elements is predicted to induce a change of structure. A surface of α A (residues α A1 [I16], α A5 [L20], and α A9 [F24]) forms a series of hydrophobic interactions with the BH3 helix (α C), while the α B helix packs against α A and the FG loop. When the α A helix is removed by Caspase 8 cleavage, a large hydrophobic surface of the BH3 helix (α C) is exposed. Interestingly, mutations of a hydrophobic surface in the first helix of BCL2 will convert BCL2 from an antiapoptotic to a proapoptotic molecule [Lee et al., 1996]. We illustrate the structural effect of the Caspase 8 cleavage event by removing the first 59 residues from the BID structure to generate a model of tBID (Figure 5a versus Figure 5b). While the displayed structures may not represent the conformation of tBID in vivo, as tBID rapidly becomes an integral membrane protein [Gross et al., 1999], the model presented for tBID in Figure 5b does suggest explanations for the changes in tBID activity and cellular localization.

It is worth noting that this type of conversion to an

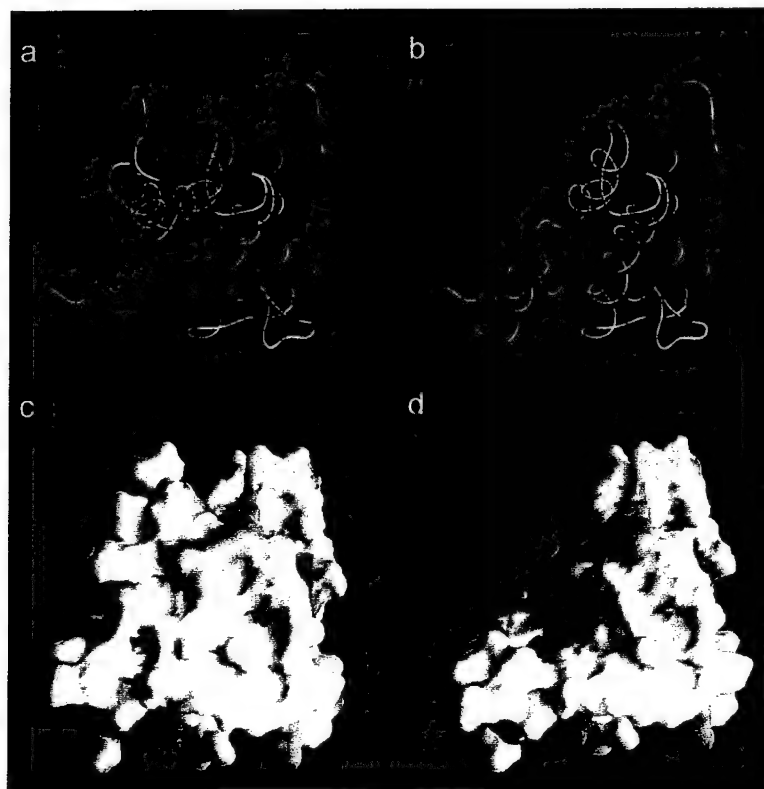


Figure 5. Predicted Structural Effects of the Caspase 8 Cleavage of BID

A ribbon diagram of (a) the full-length p22 BID versus (b) the p15 tBID model (flexible loop BC and amino terminus not shown). An analysis of the exposed hydrophobic surface for (c) BID and (d) the tBID model presented in the same orientation as in (a) and (b). Hydrophobic surfaces were generated using GRASP (Nicholls et al., 1991); here the gray and yellow indicate hydrophobic and hydrophilic surfaces, respectively.

active proapoptotic molecule is not without precedent in the BCL2 family. For both BCL2 and BCL-X_L, caspase-mediated cleavage of the flexible loop is sufficient for conversion from antiapoptotic to proapoptotic activity (Cheng et al., 1997; Clem et al., 1998). Another example can be seen in comparing the antiapoptotic BCL-X_L with the proapoptotic BCL-X_S. BCL-X_S is an alternatively spliced form of BCL-X_L that lacks helices α E- α G (see Figure 3b). The BCL-X_S therefore lacks a hydrophobic surface for the BH3 helix to pack against, allowing it unfettered access for interaction. There is also evidence that BAX can be cleaved *in vivo*, possibly as part of its cytosol to mitochondria translocation (Goping et al., 1998; Wood et al., 1998). Moreover, the amino terminus of BAX appears to be normally blocking membrane insertion, and its removal enables BAX to target mitochondrial membranes (Goping et al., 1998).

Change in Surface Character between BID and tBID
Caspase 8 cleavage results in marked changes in the character of BID surfaces. Changes in hydrophobic exposure and surface charge likely contribute to the rapid change in cellular distribution for the BID molecule observed upon cleavage. The cleavage event results in the exposure of $>200 \text{ \AA}^2$ of previously buried surface area (compare Figures 5c and 5d). The composition of surface-exposed residues in the p22 and p15 isoforms are considerably different. In addition to the change in surface hydrophobicity, the electrostatic character of the BID molecule changes markedly upon cleavage by Caspase 8. The net charge of the molecule changes from -13 in the p22 molecule to -4 in the p15 product. A striking difference can be seen in comparing the surface

charge of the two molecules; GRASP representations of the p22 and p15 molecules are shown in Figures 6a and 6b. Indeed, it seems likely that the large negative charge associated with p22 BID might be sufficient to prevent a close association with the mitochondrial outer membrane, which is among the most strongly negatively charged biological membranes (Darnell et al., 1990). The inactive p22 product is primarily ($\sim 90\%$) in the cytosol, while the p15 product is almost entirely associated with mitochondria and capable of targeting mitochondria and becoming integrated in the membrane (Gross et al., 1999). While the death inducing activities associated with the tBID molecule (i.e., cytochrome c release and activation of caspases) require an intact BH3 domain, translocation of tBID from cytosol to mitochondria can occur with a mutated BH3 (Wang et al., 1996; Luo et al., 1998). We suggest that the marked change in tBID surface electrostatics and hydrophobic exposure is likely the driving force in tBID membrane localization and subsequent membrane insertion.

Minor BID cleavage products of p13 and p11 fragments are noted following TNF and FAS activation. While p15 BID appears to be cleaved in the cytosol and translocates to mitochondria, the p13 and p11 BID fragments are only observed in mitochondria as integral membrane proteins. This suggests the p13 and p11 cleavages might be generated following integration and appear to be mediated by caspase(s) other than Caspase 8 (Gross et al., 1999). Of note, the p13 cleavage site at D75 also resides in the flexible loop and would retain the BH3 domain, whereas the p11 site at D98 would release the BH3 domain (Figure 3a). This suggests a model consistent with the predicted structure of p15 BID (Figures 5



Figure 6. A Comparison of the Change in Surface Charge between BID and the tBID Model

The overall charge in the two molecules is -13 for BID (a) and -4 for the tBID model (b). The surface electrostatic potential is coded such that regions with electrostatic potential $< -8 k_B T$ are red, while those $> +8 k_B T$ are blue (k_B , Boltzmann constant; T , absolute temperature).

and 6) in which the hydrophobic core αFG would be transmembrane, while αC (BH3 domain) would remain exposed on the surface of the mitochondria. The amino terminus of BAX also becomes more susceptible to proteolytic cleavage following membrane integration (Goping et al., 1998). There still remains the question of why Caspase 8 cleavage in a flexible loop would lead to the dissociation of helices αA and αB . With respect to this question, we do note that the hydrogen-deuterium exchange rates for αA and αB are faster than other helices in BID (see Figure 1); this may imply some conformational equilibrium between the αAB structural unit and the rest of the BID protein. There may also be involvement of third parties in the dissociation of the αAB segment.

Model of BID-Mediated Apoptosis

The exposure of the BH3 domain in BID and the proapoptotic function of the molecule is regulated through a posttranslational cleavage event. Other mechanisms of posttranslational modification regulate activities of other group I subset (Figure 3) members (Cheng et al., 1997; Gross et al., 1998). This type of regulation allows the rapid response necessary for apoptosis. Other group II constitutively active members may be regulated at the level of transcription, as predicted for EGL-1 (Conradt and Horvitz, 1998) and observed for HRK (Inohara et al., 1997). As predicted, the group II member BAD is constitutively active in its native nonphosphorylated form, while phosphorylation results in structural changes that inactivate the molecule and prevent its BH3 motif from binding BCL2 (Zha et al., 1996).

The structure of BID sheds light on its role as a death agonist and the requirements for proapoptotic action in the BCL2 family. What makes a BCL2 family member a death agonist? It appears that two criteria must be met for proapoptotic function. The first requirement is one of cellular localization. There is strong evidence for several BCL2 family members that mitochondrial translocation is a key determinant for death inducing activity. BID (Li et al., 1998; Luo et al., 1998; Gross et al., 1999) and BAX (Wolter et al., 1997; Gross et al., 1998) are inactive as

cytosolic components but induce death upon their targeting to mitochondria. The second, and perhaps more critical, criterion for death agonism is the exposure of the BH3 surface. The cleavage of BID by Caspase 8 is likely to expose a BH3 motif that is buried in the uncleaved molecule. Based on our structure predictions for the BCL2 family, some members of the "BH3 domain only" class appear to have their BH3 motif exposed or at least not under the same conformational constraints as other members of the BCL2 family. Molecules possessing an exposed BH3 motif (EGL-1, HRK, BIK, BIM, tBID) might be expected to be constitutively active death agonists. Other members of the family possessing a "hidden" BH3 motif would be activated by posttranslational modification events that expose the BH3 epitope and manifest their proapoptotic function.

Experimental Procedures

Expression of BID

Full-length mouse BID cDNA was cloned into the NcoI site of the pGEX-KG GST fusion vector (Pharmacia). Protein sequences based on this vector have 15 additional N-terminal amino acids, namely GSPGISGGGGGILDS before the M residue in the mouse BID cDNA. The expressed sequence was then gspgisgggggildsMDSEVNSGSLGAKHITDLLVFGFLQSSGCTRQEVLGRELVPVQAYWEADLEDELQTDGSAQSRFSNQGRIPDSSESQEEIHNIAHRLAQIGDEMHDNIQPTLVRLAAQFMNGSLSEEDKRNCLAKALDEVKTAFFPRDMENDKAMLIMTMLLAKKVASHAPSLRLRDVFHTTVNFINQNLFSYVRNLVRNEMD. For ^{15}N and ^{13}C samples, the following general procedure was used. BL21DE3 cells containing this fusion protein construct were grown in LB (with 100 $\mu g/ml$ ampicillin) initially, then seeded into minimal MOPS media (100 $\mu g/ml$ ampicillin) and stored as glycerol stocks in minimal MOPS media. Minimal MOPS media: 40 mM MOPS (pH 7.4) (KOH), 4 mM Tricine (pH 7.4) (KOH), 0.01 mM $FeSO_4$, 0.276 mM K_2SO_4 , 0.5 mM $CaCl_2$, 0.528 mM $MgCl_2$, 50 mM NaCl, 0.72 mM Na_2MoO_4 , 4 mM H_3BO_3 , 0.55 mM $CoCl_2$, 0.108 mM $CuSO_4$, 1.26 μM $MnCl_2$, 0.178 mM $ZnSO_4$, 1 \times vitamin solution (500 \times : in 10 ml dissolve, 16.8 mg thiamine, 11.9 mg pantothenic acid, 8 mg p-hydroxybenzoic acid, 6.9 mg p-aminobenzoic acid, 7.7 mg 2,3-dihydroxybenzoic acid), 13.1 mM K_2HPO_4 , 2 g $[U-^{13}C]$ Glucose, 1 g $^{15}NH_4Cl$. A 200 ml culture was grown in minimal MOPS media supplemented with $[U-^{13}C]$ Glucose overnight at 37°C. Ten milliliters of this overnight culture was inoculated into 1 l of minimal MOPS media (100 $\mu g/ml$ ampicillin) and grown until OD_{600} was 0.7. $[U-^{13}C]$ Glucose was added and induction was with 1 mM IPTG for 4 hr.

Cleavage and Purification from GST

Pelleted bacteria were resuspended in 14 ml (per 1 l of culture) of lysis solution (1% Triton X-100, 1 mM EDTA, PBS, and protease inhibitors [Pepstatin A, Aprotinin, Leupeptin, PMSF]). Lysis was by sonication, and debris was pelleted at 9000 rpm. The GST-BID fusion protein was captured from supernatant with GSH-agarose beads in lysis solution at 4°C with mixing for 1 hr. Beads were washed four times with lysis solution and then three times with 0.1% Triton X-100 lysis solution, followed with washing four times with sterile PBS. GST was cleaved from the fusion protein with human thrombin (600 units thrombin [Caltag] in 150 mM NaCl, 2.5 mM CaCl₂, 5 mM DTT) for 2 hr at room temperature. The digestion was stopped with 5 mM EDTA and protease inhibitors. Beads were pelleted, supernatants were pooled, and beads were washed four times with PBS, 0.1% Triton X-100, 1 mM EDTA, and protease inhibitors. BID was concentrated with an Amicon concentrator. BID was purified over a Pharmacia MonoQ anion affinity column and eluted with an increasing NaCl gradient. Mass spectra of products were correct for the sequence used. NMR samples were dialyzed and concentrated into 50 mM NaCl, 50 mM sodium phosphate (pH 6.0), 2 mM deuterated DTT, 2 mM deuterated EDTA, 0.02% sodium azide, plus 10% D₂O. Final concentrations ranged from 400 μ M to 1.4 mM; spectra were independent of concentration in this range.

NMR Spectroscopy

The assignment and structure derivation of BID were determined using standard heteronuclear NMR methods, as in McDonnell et al. (1998). Assignment made use of the following NMR experiments: HNCA, HN(CO)CA, CBCANH, CBCA(CO)NH, HNCO, HN(CA)CO, HNHA, and HCCH-TOCSY (Clare et al., 1990; Grzesiek et al., 1992; Grzesiek and Bax, 1993; Bagby et al., 1994). The high degree of redundancy in this approach allowed us to overcome significant spectral overlap to make unambiguous assignments. Automated structure calculations were performed using the program DYANA (Guntert et al., 1997). From 1000 calculated structures, the 20 with the lowest target functions were selected. The overall rmsds between the 20 structures are 0.67 ± 0.07 Å for backbone atoms and 1.42 ± 0.11 Å for all heavy atoms in the first seven helices. For the individual helices, backbone and heavy atoms rmsds range from 0.19 to 0.32 Å and 0.77 to 1.35 Å, respectively. For all residues from I16 to D195 (excluding loop BC, residues E41–E81) the values are 2.21 ± 0.50 Å and 3.16 ± 0.53 Å for backbone and all heavy atoms, respectively. The quality of calculated structures was analyzed using Procheck-NMR (Laskowski et al., 1996). From the ensemble of 20 structures, 74.5% of residues fall into the most favored regions of Ramachandran space, 90.9% of residues fall into the allowed regions, and 98.7% fall within generously allowed regions. The small number of apparently disallowed ϕ 's and ψ 's were in loops of higher rmsds and lower local NOE density. Hydrodynamic measurements on the BID molecule clearly define it as monomeric in solution. The translational diffusion coefficient was measured using pulsed-field-gradient methods (Dingley et al., 1995), and values are fully consistent with the values of hydrated monomeric BID models (from NMR-derived conformers) calculated using the bead method (de la Torre et al., 1994; McDonnell et al., 1997).

The backbone ¹⁵N relaxation parameters, comprising the rates of ¹⁵N transverse (R_2) and longitudinal (R_1) relaxation and the $\{^1\text{H}\}^{15}\text{N}$ steady-state NOE, were measured using previously described experimental protocols (Fushman et al., 1997). The "model-free" characteristics of local motions (Lipari and Szabo, 1982) were derived from these parameters using the computer program DYNAMICS (Fushman et al., 1997), taking into account the anisotropy of the overall rotational diffusion. In this study, the rotational diffusion tensor of the protein was assumed to be axially symmetric. The principal values, D_{\parallel} and D_{\perp} , and orientation (Euler angles $\{\phi, \theta, \psi\}$, $\psi = 0$ with respect to protein coordinate frame) of the rotational diffusion tensor were derived from fitting the observed relaxation rates for all core residues to the following relation, valid for small degrees of anisotropy $\epsilon \equiv D_{\parallel}/D_{\perp} - 1 < 1$: $(2R_2'/R_1 - 1)^{-1} = 3/4[1 + \epsilon \sin^2\theta (\omega_{\text{NH}})^2/[1 + (\omega_{\text{NH}})^2]]/[1 + (\omega_{\text{NH}})^2]$; θ is the angle between a given NH bond and the unique axis of the diffusion tensor; $\tau_1^{-1} \equiv 6D_{\perp}$, and ω_{NH} is the ¹⁵N resonance frequency. For this analysis, the relaxation rates were modified to subtract contributions from the

high-frequency components (P_{HF}) of local motion (Fushman and Cowburn, 1998) as follows (Farrow et al., 1995): $R_1' = R_1 - 6.25P_{\text{HF}}$; $R_2' = R_2 - 5.39P_{\text{HF}}$, where $P_{\text{HF}} = (\gamma_{\text{N}}/\gamma_{\text{H}})(1 - \text{NOE})R_1/5$ and γ_{N} and γ_{H} are gyromagnetic ratios for ¹⁵N and ¹H. For the derivation of the relative orientation of individual helices with respect to the overall diffusion tensor, the average orientation of each backbone amide was calculated based on the NMR-derived bundle of 20 structures. The orientation of the principal axes of the rotational diffusion tensor was derived using the R_2/R_1 ratios (Copie et al., 1998). The analysis, applied to 20 generated BID structures with the lowest target function, yielded the following averaged characteristics of the overall rotation: $\tau_c = 9.44 \pm 0.03$ ns, $D_{\parallel}/D_{\perp} = 1.18 \pm 0.01$, $\theta = 65^\circ \pm 6^\circ$, and $\phi = 27^\circ \pm 8^\circ$. The values for individual amide orientations were averaged for each helical segment, giving an average relative orientation for each helix.

Structure Prediction for Family Members

For the sequence alignment for the BCL2 family, the program CLUSTALW (Thompson et al., 1994) was used initially. However, the low level of similarity often necessitated aligning sequences by hand. GenBank accession numbers are as follows: BID, g1669514; BCL-X_L, g278955; BCL2, g231632; CED-9, g1168881; BCL-W, g2493278; BAX, g278945; BAK, g2493274; BAD, g2493287; EGL-1, g3133315; BIK, g1235989. The aligned sequences were then subjected to secondary structure prediction methods. A number of programs were used for this analysis. Where possible, the results displayed in Figure 3b represent a consensus of the multiple approaches used. The following programs contributed to BCL2 family secondary structure predictions: PSA (Stultz et al., 1993), PHDsec (Rost et al., 1994), PREDATOR (Frishman and Argos, 1996), DSC (King and Sternberg, 1996), SOPMA (Geourjon and Deleage, 1995), and BCM PSSP (Solovyev and Salamov, 1994).

Acknowledgments

We are grateful to Dr. Frank Löhr for some of the pulse sequences used; Dr. Jie Zheng for his help in early aspects of the project; and Dr. Sean Cahill for technical support. D. C. thanks Professor John Kuriyan for comments. This work was supported by NIH grants GM-47021 (D. C.) and CA-50239 (S. J. K.).

Received December 24, 1998; revised January 26, 1999.

References

- Adams, J.M., and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science* 281, 1322–1326.
- Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., et al. (1997). Inhibition of Bax channel-forming activity by Bcl-2. *Science* 277, 370–372.
- Bagby, S., Harvey, T.S., Kay, L.E., Eagle, S.G., Inouye, S., and Ikura, M. (1994). Unusual helix-containing Greek keys in development-specific Ca²⁺-binding protein S. ¹H, ¹⁵N, and ¹³C assignments and secondary structure determined with the use of multidimensional double and triple resonance heteronuclear NMR spectroscopy. *Biochemistry* 33, 2409–2421.
- Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J., et al. (1995). Bik, a novel death-inducing protein, shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* 11, 1921–1928.
- Broadhurst, R.W., Hardman, C.H., Thomas, J.O., and Laue, E.D. (1995). Backbone dynamics of the A-domain of HMG1 as studied by ¹⁵N NMR spectroscopy. *Biochemistry* 34, 16608–16617.
- Chao, D.T., and Korsmeyer, S.J. (1998). BCL-2 family: regulators of cell death. *Annu. Rev. Immunol.* 16, 395–419.
- Cheng, E.H., Kirsch, D.G., Clem, R.J., Ravi, R., Kastan, M.B., Bedi, A., Ueno, K., and Hardwick, J.M. (1997). Conversion of Bcl-2 to a Bax-like death effector by caspases. *Science* 278, 1966–1968.

- Chinnaiyan, A.M., O'Rourke, K., Lane, B.R., and Dixit, V.M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* 275, 1122-1126.
- Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G., and Lutz, R.J. (1995). A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *EMBO J.* 14, 5589-5596.
- Chou, J.J., Li, H., Salvesen, G.S., Yuan, J., and Wagner, G. (1999). Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell* 96, this issue, 615-624.
- Clem, R.J., Cheng, E.H., Karp, C.L., Kirsch, D.G., Ueno, K., Takahashi, A., Kastan, M.B., Griffin, D.E., Earnshaw, W.C., Veluona, M.A., and Hardwick, J.M. (1998). Modulation of cell death by Bcl-X_L through caspase interaction. *Proc. Natl. Acad. Sci. USA* 95, 554-559.
- Clore, G.M., Bax, A., Driscoll, P.C., Wingfield, P.T., and Gronenborn, A.M. (1990). Assignment of the side-chain ¹H and ¹³C resonances of interleukin-1 beta using double- and triple-resonance heteronuclear three-dimensional NMR spectroscopy. *Biochemistry* 29, 8172-8184.
- Conradt, B., and Horvitz, H.R. (1998). The *C. elegans* protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell* 93, 519-529.
- Copie, V., Tomita, Y., Akiyama, S.K., Aota, S., Yamada, K.M., Venable, R.M., Pastor, R.W., Krueger, S., and Torchia, D.A. (1998). Solution structure and dynamics of linked cell attachment modules of mouse fibronectin containing the RGD and synergy regions: comparison with the human fibronectin crystal structure. *J. Mol. Biol.* 277, 663-682.
- Damell, J., Lodish, H., and Baltimore, D. (1990). *Molecular Cell Biology* (New York: W. H. Freeman and Company).
- de la Torre, J.G., Navarro, S., Martinez, M.C.L., Diaz, F.G., and Cascales, J.J.L. (1994). HYDRO: a computer program for the prediction of hydrodynamic properties of macromolecules. *Biophys. J.* 67, 530-531.
- Dingley, A.J., Mackay, J.P., Chapman, B.E., Morris, M.B., Kuchel, P.W., Hambly, B.D., and King, G.F. (1995). Measuring protein self-association using pulsed-field-gradient NMR spectroscopy: application to myosin light chain 2. *J. Biomol. NMR* 6, 321-328.
- Ellis, R.E., Yuan, J.Y., and Horvitz, H.R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* 7, 663-698.
- Evans, S.V. (1993). SETOR: hardware lighted three-dimensional solid model representations of macromolecules. *J. Mol. Graph.* 11, 134-138.
- Farrow, N., Zhang, O., Szabo, A., Torchia, D., and Kay, L. (1995). Spectral density function mapping using ¹⁵N relaxation data. *J. Biomol. NMR* 6, 153-162.
- Frishman, D., and Argos, P. (1996). Incorporation of non-local interactions in protein secondary structure prediction from the amino acid sequence. *Protein Eng.* 9, 133-142.
- Fushman, D., and Cowburn, D. (1998). Model-independent analysis of ¹⁵N chemical shift anisotropy from NMR relaxation data. Ubiquitin as a test example. *J. Am. Chem. Soc.* 120, 7109-7110.
- Fushman, D., Cahill, S., and Cowburn, D. (1997). The main chain dynamics of the dynamin pleckstrin homology (PH) domain in solution: analysis of ¹⁵N relaxation with monomer/dimer equilibration. *J. Mol. Biol.* 266, 173-194.
- Geourjon, C., and Deleage, G. (1995). SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Comput. Appl. Biosci.* 11, 681-684.
- Goping, I.S., Gross, A., Lavoie, J.N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J., and Shore, G.C. (1998). Regulated targeting of BAX to mitochondria. *J. Cell Biol.* 143, 207-215.
- Gross, A., Jockel, J., Wei, M.C., and Korsmeyer, S.J. (1998). Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J.* 17, 3878-3885.
- Gross, A., Yin, X.-M., Wang, K., Wei, M.C., Jockel, J., Milliman, C., Erjument-Bromage, H., Tempst, P., and Korsmeyer, S.J. (1999). Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-X_L prevents this release but not TNF-R1/Fas death. *J. Biol. Chem.* 274, 1156-1163.
- Grzesiek, S., and Bax, A. (1993). Amino acid type determination in the sequential assignment procedure of uniformly ¹³C/¹⁵N-enriched proteins. *J. Biomol. NMR* 3, 185-204.
- Grzesiek, S., Dobeli, H., Gentz, R., Garotta, G., Labhardt, A.M., and Bax, A. (1992). ¹H, ¹³C, and ¹⁵N NMR backbone assignments and secondary structure of human interferon-gamma. *Biochemistry* 31, 8180-8190.
- Guntert, P., Mumenthaler, C., and Wüthrich, K. (1997). Torsion angle dynamics for NMR structure calculation with the new program DYANA. *J. Mol. Biol.* 273, 283-298.
- Guntert, P., Braun, W., and Wüthrich, K. (1991). Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS, and GLOMSA. *J. Mol. Biol.* 217, 517-530.
- Hegde, R., Srinivasula, S.M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E.S. (1998). Bkl, a BH3-containing mouse protein that interacts with Bcl-2 and Bcl-X_L, is a potent death agonist. *J. Biol. Chem.* 273, 7783-7786.
- Inohara, N., Ding, L., Chen, S., and Nunez, G. (1997). harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-X_L. *EMBO J.* 16, 1686-1694.
- King, R.D., and Sternberg, M.J. (1996). Identification and application of the concepts important for accurate and reliable protein secondary structure prediction. *Protein Sci.* 5, 2298-2310.
- Laskowski, R.A., Rullmann, J.A., MacArthur, M.W., Kaptein, R., and Thornton, J.M. (1996). AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR* 8, 477-486.
- Lee, L.C., Hunter, J.J., Mujeeb, A., Turck, C., and Parslow, T.G. (1996). Evidence for alpha-helical conformation of an essential N-terminal region in the human Bcl2 protein. *J. Biol. Chem.* 271, 23284-23288.
- Li, H., Zhu, H., Xu, C.J., and Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94, 491-501.
- Lipari, G., and Szabo, A. (1982). Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. *J. Am. Chem. Soc.* 104, 4559-4570.
- Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell* 94, 481-490.
- McDonnell, J.M., Fushman, D., Cahill, S.M., Sutton, B.J., and Cowburn, D. (1997). Solution structures of FcεRI α-chain mimics: a β-hairpin peptide and its retroenantiomer. *J. Am. Chem. Soc.* 119, 5321-5328.
- McDonnell, J.M., Fushman, D., Cahill, S.M., Zhou, W., Wolven, A., Wilson, C.B., Nelle, T.D., Resh, M.D., Wills, J., and Cowburn, D. (1998). Solution structure and dynamics of the bioactive retroviral M domain from Rous sarcoma virus. *J. Mol. Biol.* 279, 921-928.
- Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettekheim, D., Chang, B.S., Thompson, C.B., Wong, S.L., et al. (1996). X-ray and NMR structure of human Bcl-X_L, an inhibitor of programmed cell death. *Nature* 381, 335-341.
- Murzin, A.G., Brenner, S.E., Hubbard, T., and Chothia, C. (1995). SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J. Mol. Biol.* 274, 536-540.
- Nicholls, A., Sharp, K.A., and Honig, B. (1991). Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins* 11, 281-296.
- O'Connor, L., Strasser, A., O'Reilly, L.A., Hausmann, G., Adams, J.M., Cory, S., and Huang, D.C. (1998). Bim: a novel member of the Bcl-2 family that promotes apoptosis. *EMBO J.* 17, 384-395.
- Oltvai, Z.N., Millman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74, 609-619.
- Raff, M.C. (1992). Social controls on cell survival and cell death. *Nature* 356, 397-400.
- Rost, B., Sander, C., and Schneider, R. (1994). PHD—an automatic

- mail server for protein secondary structure prediction. *Comput. Appl. Biosci.* 10, 53-60.
- Sattler, M., Liang, H., Nettlesheim, D., Meadows, R.P., Harlan, J.E., Eberstadt, M., Yoon, H.S., Shuker, S.B., Chang, B.S., Minn, A.J., et al. (1997). Structure of Bcl-x_L-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275, 983-986.
- Schlesinger, P.H., Gross, A., Yin, X.M., Yamamoto, K., Saito, M., Waksman, G., and Korsmeyer, S.J. (1997). Comparison of the ion channel characteristics of proapoptotic BAX and antiapoptotic BCL-2. *Proc. Natl. Acad. Sci. USA* 94, 11357-11362.
- Solovyev, V.V., and Salamov, A.A. (1994). Predicting alpha-helix and beta-strand segments of globular proteins. *Comput. Appl. Biosci.* 10, 661-669.
- Strasser, A., Huang, D.C., and Vaux, D.L. (1997). The role of the bcl-2/ced-9 gene family in cancer and general implications of defects in cell death control for tumorigenesis and resistance to chemotherapy. *Biochim. Biophys. Acta* 1333, F151-F178.
- Stultz, C.M., White, J.V., and Smith, T.F. (1993). Structural analysis based on state-space modeling. *Protein Sci.* 2, 305-314.
- Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* 267, 1456-1462.
- Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680.
- Thomberry, N.A., and Lazebnik, Y. (1998). Caspases: enemies within. *Science* 281, 1312-1316.
- Tjandra, N., Garrett, D.S., Gronenborn, A.M., Bax, A., and Clore, G.M. (1997). Defining long range order in NMR structure determination from the dependence of heteronuclear relaxation times on rotational diffusion anisotropy. *Nat. Struct. Biol.* 4, 443-449.
- Wang, K., Yin, X.M., Chao, D.T., Millman, C.L., and Korsmeyer, S.J. (1996). BID: a novel BH3 domain-only death agonist. *Genes Dev.* 10, 2859-2869.
- Wang, K., Gross, A., Waksman, G., and Korsmeyer, S.J. (1998). Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. *Mol. Cell. Biol.* 18, 6083-6089.
- Wishart, D., Sykes, B.D., and Richard, F.M. (1992). The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* 31, 1647-1651.
- Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G., and Youle, R.J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J. Cell Biol.* 139, 1281-1292.
- Wood, D.E., Thomas, A., Devi, L.A., Berman, Y., Beavis, R.C., Reed, J.C., and Newcomb, E.W. (1998). Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene* 17, 1069-1078.
- Wüthrich, K. (1986). *NMR of Proteins and Nucleic Acids* (New York: John Wiley and Sons).
- Yang, E., Zha, J., Jockel, J., Boise, L.H., Thompson, C.B., and Korsmeyer, S.J. (1995). Bad, a heterodimeric partner for Bcl-X_L and Bcl-2, displaces Bax and promotes cell death. *Cell* 80, 285-291.
- Yin, X.M., Oltvai, Z.N., and Korsmeyer, S.J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* 369, 321-323.
- Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X_L. *Cell* 87, 619-628.
- Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G., and Korsmeyer, S.J. (1997). BH3 domain of BAD is required for heterodimerization with BCL-X_L and pro-apoptotic activity. *J. Biol. Chem.* 272, 24101-24104.

Protein Data Bank ID Code

The protein BID has been deposited in the Protein Data Bank under ID code 1ddb.